Laboratory Exercise # 6: Gram Stain

Purpose:

The student will perform the staining procedure called the Gram Stain, which is used to classify bacteria into two groups.

Introduction:

Simple staining (the use of a single stain) allows a microbiologist to observe the morphology (shape) and arrangement of bacteria. In order to classify bacteria into different groups a differential staining procedure must be done. A differential stain involves the use of two or more stains. Depending on the components of the bacterial cell wall or outer layers, the bacteria will either retain the primary stain or have the primary stain removed in a decolorizing step and then retain the secondary stain.

The gram stain is the most common differential stain used in the microbiology laboratory to categorize bacteria. The primary stain is the cationic dye Crystal violet and the secondary stain is the cationic dye Safranin. Since both stains are cationic, they are both attracted to the negatively charged particles in the bacterial cell wall. So what causes some bacteria to retain the Crystal violet stain while others are decolorized by the alcohol and then pick up the Safranin stain?

The current theory behind gram staining is that the crystal violet enters the thick layer of the peptidoglycan of the gram positive cell wall. Then the iodine acts as a mordant and binds to the crystal violet. This large complex is caught within the complex of the peptidoglycan and isn’t washed away by the alcohol, used as a decolorizer. In gram negative bacteria the crystal violet-iodine complex is mainly taken up by the lipids within the outer membrane. Lipids are soluble in alcohol so the stain is washed away with the alcohol decolorizer. Then the thin layer of peptidoglycan (found below the lipid layer) can be stained with the secondary stain, Safranin.

Materials:

Crystal violet stain  Inoculating loop
Gram’s iodine     Wax marker
Gram’s alcohol    Bunsen burner
Safranin stain   Bibulous paper
Glass slides

Bacterial cultures for use with this exercise are listed in the next laboratory exercise.
**Procedure:**

1. Make your bacterial smear and heat fix it as done for the simple stain procedure.
2. Let the smear cool totally.
3. Cover the smear with Crystal violet stain for one (1) minute.
4. Rinse with water from the squirt bottle.
5. Cover the smear with Gram’s iodine for one (1) minute.
6. Rinse with water from the squirt bottle.
7. Decolorize with Gram’s alcohol. Hold slide at an angle and drip on alcohol, one drop at a time until stain stops running off. (Three to four drops should be sufficient.)
8. Rinse with water from the squirt bottle.
9. Counterstain with Safranin stain for 30 seconds.
10. Rinse with water from the squirt bottle.
11. Blot the smear dry with bibulous paper.
12. Examine the smear under 100X after first focusing on 10X.

**Questions:**

1. Which stain is considered the primary stain in this procedure?
2. Which stain is considered the secondary stain in this procedure?
3. Why is Gram’s iodine called a mordant?
4. What happens if you over decolorize in the Gram stain procedure?
5. Why do some bacteria turn purple and others turn red when you use this staining procedure?